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Synergistic Neutralization of HIV-1 by Human Monoclonal Antibodies Against the V3 Loop and the CD4-Binding Site of gp120

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ABSTRACT

Two distinct regions or epitope clusters of human immunodeficiency virus type 1 (HIV-1) gp120 have been shown to elicit neutralizing antibodies: the V3 loop and the CD4-binding site. We have isolated neutralizing human monoclonal antibodies (HuMAbs) against conserved epitopes in both of these regions. In this study, we demonstrate that an equimolar mixture of two of these HuMAbs, one directed against the V3 loop and the other against the CD4-binding site, neutralizes HIV-1 at much lower concentrations than does either of the individual HuMAbs. Mathematical analysis of this effect suggests cooperative neutralization of HIV-1 by the two HuMAbs and demonstrates a high level of synergy, with combination indices (CIs) of 0.07 and 0.16 for 90% neutralization of the MN and SF-2 strains, respectively. The dose reduction indices (DRIs) for each of the two HuMAbs at 99% neutralization range approximately from 10 to 150. A possible mechanism for this synergism is suggested by binding studies with recombinant gp160 of the MN strain; these show enhanced binding of the anti-CD4 binding site HuMAb in the presence of the anti-V3 loop HuMAb. These results demonstrate the advantage of including both V3 loop and CD4-binding site epitopes in a vaccine against HIV-1 and indicate that combinations of HuMAbs against these two sites may be particularly effective in passive immunotherapy against the virus.

INTRODUCTION

TWO REGIONS OR EPITOPE CLUSTERS of human immunodeficiency virus type 1 (HIV-1) gp120 have been shown to elicit neutralizing antibodies against the virus. One of these regions is the hypervariable V3 loop (aa 296–331), an immunodominant epitope cluster eliciting predominantly strain-specific antibodies in humans and experimental animals.[1–5] There was the initial concern that the hypervariability of the V3 loop would prevent the design of a rational vaccine or immunotherapy based on this epitope. However, LaRosa et al.[6] have shown recently that the V3 loop is less variable among primary HIV-1 isolates than originally thought, and that anti-V3 antibodies elicited in rodents against the tip of the loop possess broad HIV-1 strain specificity.[7]

The other epitope cluster of gp120 that elicits neutralizing antibodies is the CD4-binding site. Recent evidence indicates that this site is formed by noncontiguous protein sequences from multiple regions of gp120 that are relatively conserved among HIV-1 isolates;[8] however, the precise structure of the CD4 binding site and its contact residues have yet to be defined. In a number of vaccination studies using recombinant *env* proteins, insignificant levels of antibodies against this site were produced, suggesting that this region is not very immunogenic.[9,10] However, neutralizing antibodies against this site have been raised in rodents,[11–13] using either recombinant gp120 or linear peptides adjacent to one of the loops apparently forming the CD4-binding site. Recently, we[14] and others[15–18] have demonstrated that HIV-1-infected humans can produce neutralizing antibodies against the CD4-binding site. From a healthy, seropositive

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hemophiliac, we isolated a HuMAb, 1125H (γ1, κ), that recognizes a conserved, conformational epitope mapping in the CD4-binding region. This HuMAb exhibits potent neutralizing activity against a variety of HIV-1 strains, including MN and SF-2.[14]

In this report, we describe an additional neutralizing HuMAb, 4117C,[19] against a relatively conserved epitope of the V3 loop. We also document that 4117C and 1125H synergistically neutralize HIV-1 such that 10- to 100-fold lower concentrations of each HuMAb is required when combined at a 1:1 ratio than when used alone to achieve the same high levels of neutralization. We believe that this is the first observation of synergistic neutralization of HIV-1 by antibodies specific for the V3 loop and CD4 binding site.

MATERIALS AND METHODS

Isolation of HuMAb 4117C

HuMAb 4117C was derived by Epstein-Barr virus (EBV)-transformation of peripheral blood mononuclear cells from an asymptomatic, seropositive hemophiliac essentially as described[14] except that $V3_{MN}$ peptide (aa 305–328) rather than recombinant gp160 was used in the primary screening by enzyme-linked immunosorbent assay (ELISA) for anti-*env* antibody-producing cultures. The cells were cloned by limiting dilution and were shown to be monoclonal by Southern blot analysis using an immunoglobulin J_H gene probe as described.[14]

Characterization of HuMAb 4117C

Isotyping, immunoblot analysis, immunofluorescence assays against virus-infected cells, and ELISAs against V3 loop peptides were carried out as described.[14] Epitope mapping was done using an epitope scanning kit (Cambridge Research Biochemicals, Valley Stream, NY) according to the manufacturer's protocol. Specifically, overlapping nine amino acid peptides spanning both the MN and SF-2 V3 loop sequences (aa 305–328) were reacted with supernatant from the 4117C cell line that was diluted 1/100 in pre-coat buffer (2% bovine serum albumin (BSA), 0.1% Tween-20, 0.01M phosphate-buffered saline (PBS), pH 7.4) containing 0.1% NaN_3. The bound 4117C HuMAb was detected with a 1/3000 dilution of goat anti-human IgG conjugated with horseradish peroxidase (Zymed) in conjugate diluent (1% fetal calf serum (FCS), 0.1% Tween-20, 0.1% sodium caseinate, 0.01M PBS, pH 7.4) followed by washing and addition of substrate, 2,2′-azino-di[3-ethyl-benzthiazoline sulfonate].

Preparation of affinity-purified chimpanzee antibodies against the V3 loop

Serum was obtained from chimpanzee #433 that had been hyperimmunized with $V3_{BRU}$ peptide as described.[9] The serum was diluted 1/10 in 10 mM Tris, pH 7.5 and the anti-$V3_{BRU}$ antibodies were purified on an AH-Sepharose column to which $V3_{BRU}$ peptide had been covalently attached using glutaraldehyde. After washing the column with 500 mM NaCl and 10 mM Tris, pH 7.5, the anti-$V3_{BRU}$ antibodies were eluted with 100 mM glycine, pH 2.5. Eluted fractions were immediately neutralized using 1 M Tris-HCl, pH 8.0 and were dialyzed against PBS.

Neutralization assay

Our 24-hour fluorescent focus assay for HIV neutralization has been described.[14,20] Briefly, HuMAbs purified on protein A filters as reported[14] were preincubated at various concentrations with 10^4 infectious units of HIV, as independently determined by end-point dilution of virus. The HuMAb/virus mixtures were added to 10^5 H9 cells, incubated for 24 hours, and then the number of virus-infected cells was assessed by immunofluorescence. In these studies, the latter was accomplished by staining of fixed virus-infected cells with rat anti-*nef* serum followed by rabbit anti-rat IgG conjugated to FITC; immunofluorescent cells were counted under a fluorescence microscope. Using an antibody against a nonstructural antigen, *nef*, allows rapid detection of infection and prevents background labeling of input viral antigens.

This assay reproducibly and accurately measures the ability of antibodies to block the first round of infection of cells with HIV. As early as 16 hours following infection, synthesis of *nef* can be easily detected in this assay. Orders of magnitude higher levels of infectious virions (10^4 to 10^5) are used in this assay, as compared with more conventional several-day assays (~50 infectious particles), in order to allow detection of infection after 24 hours. Under these conditions, 1–5% of cells are usually infected at this time. Thus, more antibody typically is required in this assay to neutralize a given percentage of virions than is required in several-day assays using much less virus (Pinter et al., unpublished results). Nevertheless, neutralizing ability in this assay correlates well with that found in various several-day assays.[9,20] Advantages of this assay are its rapidity, its accuracy and reproducibility, and the possibility of easily obtaining neutralization curves such as those shown in Figure 1 (i.e., % neutralizations at different HuMAb concentrations) in a single assay.

Mathematical analysis of neutralization data

Neutralization data were analyzed by a computer program[21] that fits the data to the median-effect equation, determines a correlation coefficient indicating goodness of fit of the data to the median-effect equation, and quantitates the amount of synergism observed. The median-effect equation is: % neutralization/(100% − % neutralization) = $(D/D_m)^m$, where D is the concentration of HuMAb required to produce a given percent neutralization, D_m is the concentration of HuMAb required to produce 50% neutralization, and m is a coefficient defining the shape of the neutralization curve.[22]

The amount of synergism is given by a combination index (CI) value that varies inversely with the amount of synergism observed. CI (at a given % neutralization) = $(D)_1/(D_x)_1 + (D)_2/(D_x)_2 + \alpha(D)_1(D)_2/(D_x)_1(D_x)_2$, where $(D)_1$ and $(D)_2$ are the concentrations of HuMAbs 1 and 2, respectively, in the mixture used to achieve a given percent neutralization and $(D_x)_1$ and $(D_x)_2$ are the concentrations of HuMAbs 1 and 2, respectively, used alone to achieve that same percent neutralization. For HuMAbs having different modes of action or acting

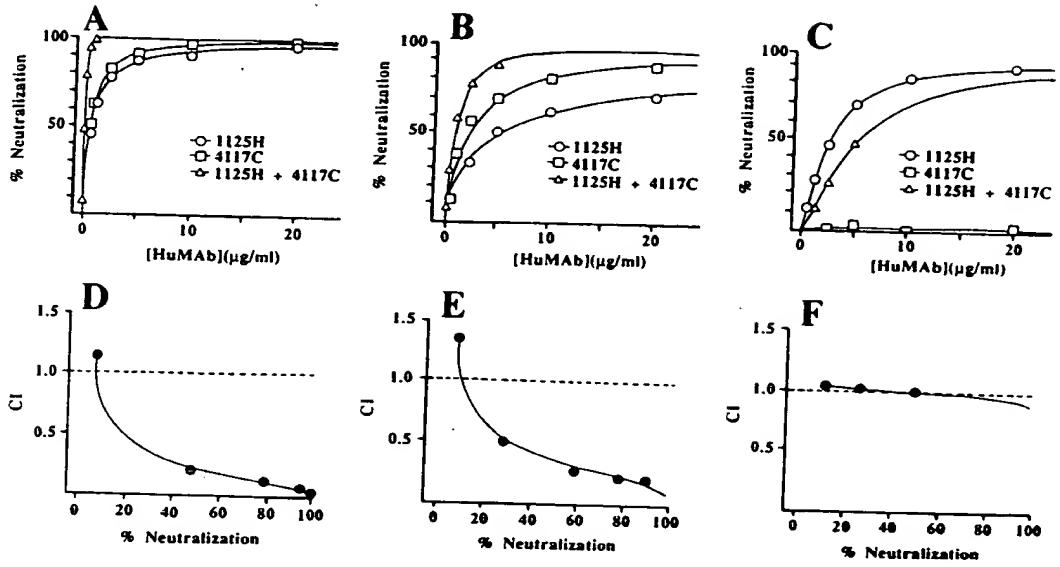

FIG. 1. Neutralization of different HIV-1 strains by two HuMAbs used individually or combined in a 1:1 ratio and plots of combination index (CI) values versus % neutralization calculated from the results. Neutralization assays were done as described[14,20] using 1×10^4 infectious units of virus per 1×10^5 H9 cells. Shown are neutralization of (A) MN strain, (B) SF-2 strain, and (C) IIIB strain. CI versus % neutralization plots were calculated from (D) MN neutralization data, (E) SF-2 neutralization data, and (F) IIIB neutralization data.

independently (mutually nonexclusive), $\alpha = 1$, whereas for HuMAbs having the same or similar modes of action (mutually exclusive), $\alpha = 0$.[22] In our calculations, we assume that $\alpha = 1$.

Simultaneous two HuMAb binding assay

Recombinant $gp160_{MN}$ (Pasteur Vaccins) that is analogous to the $gp160_{BRU}$ (construct #1163) previously described[23] was used to coat ELISA plates at 10 ng/well in PBS. All washes were done in PBS. A constant amount of each biotinylated HuMAb, prepared as described,[14] was combined with different concentrations of unlabeled HuMAbs in 1% BSA/PBS at a constant volume, and 50 μl of these mixtures was plated in quintuplicate onto $gp160_{MN}$-coated plates. The final concentration of the biotinylated HuMAbs was 0.2 μg/ml, while the final concentrations of the unlabeled HuMAbs ranged from 0.03 to 30 μg/ml. The bound biotinylated HuMAbs were detected with streptavidin coupled to alkaline phosphatase followed by disodium *p*-nitrophenyl phosphate. Control absorbance was defined as the absorbance (405 nm) obtained in the assay when each biotinylated HuMAb (0.2 μg/ml) was used alone.

RESULTS

From a healthy, seropositive hemophiliac, we isolated a HuMAb, 4117C (γ1, λ), against a relatively conserved epitope of the V3 loop. The specificity and broad strain reactivity of this HuMAb have been established by immunoblot analysis, immunofluorescence assays against virus-infected cells, and ELISAs against V3 loop peptides. The latter assays demonstrated binding of 4117C to V3 peptides from several divergent HIV-1 strains, including MN, SF-2, and some African isolates, but not IIIB (data not shown). The strain specificity of 4117C implied that it was directed against an epitope near the tip of the V3 loop. This conclusion was confirmed by epitope mapping; 4117C recognized a minimum of seven amino acids at the tip of the loop, i.e., IXIGPGR. Both the 4117C HuMAb and the previously described 1125H HuMAb[14] possess potent neutralizing activity against the MN and SF-2 strains of HIV-1 (Figs. 1A, B).

When combined, these two HuMAbs synergistically neutralize HIV-1. Figure 1A shows that appoximately 0.7 μg/ml of either HuMAb alone is required to achieve 50% neutralization of the MN strain, while approximately 0.15 μg/ml of the two HuMAbs combined in a 1:1 ratio achieves this level of neutralization. At higher levels of neutralization, the effect is even more dramatic. To obtain greater than 90% neutralization of the MN strain, over 4 μg/ml of each HuMAb alone is required, whereas this level of neutralization is achieved by approximately 0.4 μg/ml of the combination of HuMAbs. Synergistic neutralization was also observed against the SF-2 strain with this pair of HuMAbs (Fig. 1B), although the effect is not quite as profound as with the MN strain. Preliminary results indicate that this reduced synergism may be due to heterogeneity in the SF-2 virus population with a consequent difference in binding affinities of the two HuMAbs to different viral subpopulations.

The neutralization data shown in Figures 1A–C were analyzed by a computer program[21] that fits the data to the median-effect equation, evaluates the goodness of fit, indicates whether there is cooperativity involved in the synergistic interaction, and quantitates the amount of synergism observed. The curves seen in these figures were generated by the program as the best fit to the data points shown.

Synergism may be quantitated by determination of a combination index (CI) value. When the CI value is less than 1,

synergism is indicated, and the greater the synergism, the lower the CI value.[22] CI values of 1 indicate additivity, i.e., neither synergistic nor antagonistic interaction. The computer program discussed above was used to calculate CI values across a range of neutralization levels from the neutralization curves shown in Figures 1A–C, the results are shown in Figures 1D–F, respectively. Potent synergism is seen at physiologically significant neutralization levels (>50% neutralization) against both the MN and SF-2 strains (Figs. 1D, E). The greatest synergism (lowest CI values) is observed at the highest neutralization levels. The synergism observed against the MN strain is as high as any yet observed between any two drugs or MAbs, i.e., +4 synergism (CI = 0.01 to 0.2), whereas that for the SF-2 strain is a +3 synergism (CI = 0.2 to 0.4).[24]

Table 1 shows other parameters calculated from the results shown in Figures 1A,B. The linear correlation coefficient, r, indicates the goodness of fit of the data to the median-effect equation, where a value of 1 indicates a perfect fit. The r values for MN neutralization are > 0.99, and those for SF-2 neutralization are > 0.98. The slope of the median-effect plot, m, signifies the shape of the neutralization curve, i.e., hyperbolic for $m = 1$ or sigmoidal for $m > 1$. Values of m that are significantly greater than 1 indicate cooperativity.[24] Thus, the fact that the m values for the combination of HuMAbs approach 2, whereas the m values for each HuMAb alone are approximately 1, indicates cooperativity between this pair of HuMAbs in neutralizing HIV-1.

The dose reduction index (DRI) is the concentration of either of the HuMAbs alone required to effect a given level of neutralization divided by the concentration of that HuMAb in combination required to effect that level of neutralization. In these experiments, the latter concentration is half of the HuMAb mixture's total concentration. The DRI increases with the level of neutralization achieved. For instance, at 90% neutralization, the DRIs for the two HuMAbs and two viral strains range from 7 to 37, while at 99% neutralization, the DRIs range from 14 to 164 (Table 1).

Additional experiments have been performed to address the generality and specificity of this synergistic neutralization of HIV-1. We have observed synergistic neutralization of the MN and SF-2 strains by 4117C combined with another neutralizing, anti-CD4 binding site HuMAb of different epitope specificity from that of 1125H (manuscript in preparation). We have also observed synergistic neutralization of the IIIB strain of HIV-1 by 1125H combined with neutralizing, affinity-purified anti-V3 loop antibodies from a hyperimmunized chimpanzee. The latter antibodies are strain specific[9] and, therefore, against a different epitope(s) than the 4117C anti-V3 HuMAb. Thus, our results show that neutralizing antibodies against at least two different V3 loop epitopes and two different CD4-binding site epitopes can participate in synergistic neutralization, and this synergism has been observed with three different HIV-1 strains. As a specificity control, neutralization of the IIIB strain of HIV-1 by the combination of 1125H and 4117C was assessed. Since 4117C does not recognize nor neutralize the IIIB strain (Fig. 1C), it would be expected to have no effect on the neutralization of IIIB by 1125H. Indeed, Figure 1C shows that this is the case. The total HuMAb concentration at which a given percent neutralization is attained by the 1:1 combination of the two HuMAbs is precisely twice the concentration of 1125H alone required to achieve that level of neutralization. Thus, additivity (i.e., neither synergism nor antagonism) is seen in this experiment (Fig. 1F). Also, an anti-gp41 HuMAb with high affinity but no neutralizing activity, 31710B,[14] had no effect on neutralization by either neutralizing anti-V3 loop or anti-CD4 binding site HuMAbs. These findings indicate that the synergistic neutralization observed is specific for antibodies that bind to and neutralize a particular strain of HIV-1.

A possible mechanism for this synergism is suggested by binding studies with recombinant gp160 of MN strain. In these experiments, one or the other of the HuMAbs was labeled with biotin so that its binding to $gp160_{MN}$ could be specifically followed. The binding of each biotinylated HuMAb to $gp160_{MN}$ could be inhibited by its unlabeled form (Fig. 2), confirming the binding activity and specificity of the HuMAbs. The binding of biotinylated 1125H to gp160 is significantly increased in the presence of optimal concentrations of the 4117C HuMAb; in the experiment shown in Figure 2, the increase is approximately

TABLE 1. PARAMETERS CALCULATED FROM NEUTRALIZATION DATA SHOWN IN FIG. 1A AND 1B USING THE CHOU AND CHOU COMPUTER PROGRAM

HuMAbs	r[b]	m[c]	*DRI*[a] *at % neutralization =*					
			50	*70*	*90*	*95*	*97*	*99*
MN neutralization								
1125H	0.992	0.97 ± 0.06[d]	9.6	16.1	36.5	57.5	79.4	156.5
4117C	0.997	1.23 ± 0.05	9.4	13.0	21.7	28.9	35.5	54.4
1125H + 4117C	0.992	2.33 ± 0.17	NA					
SF-2 neutralization								
1125H	0.992	0.80 ± 0.07	8.9	15.2	35.8	57.4	80.5	163.5
4117C	0.982	1.15 ± 0.11	4.3	5.3	7.4	8.9	10.2	13.5
1125H + 4117C	0.992	1.61 ± 0.12	NA					

[a]Dose reduction index = $(D_x)_n/(D)_n$. (See Materials and Methods.)
[b]Linear correlation coefficient.
[c]Slope of the neutralization curve.
[d]Standard error of the mean.
NA = not applicable.

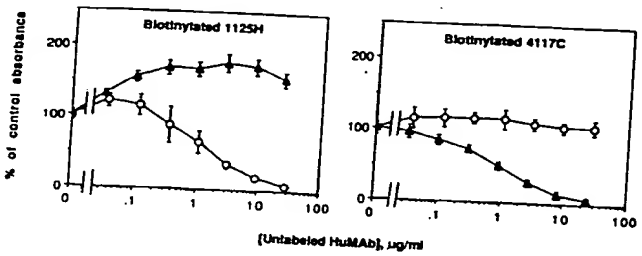

FIG. 2. Simultaneous two HuMAb binding assay using recombinant gp160 of MN strain as antigen. Control absorbance was defined as the absorbance (405 nm) obtained in the assay when each biotinylated HuMAb (0.2 μg/ml) was used alone. Unlabeled HuMAbs were 1125H (○) and 4117C (▲). Error bars represent standard deviation from the mean.

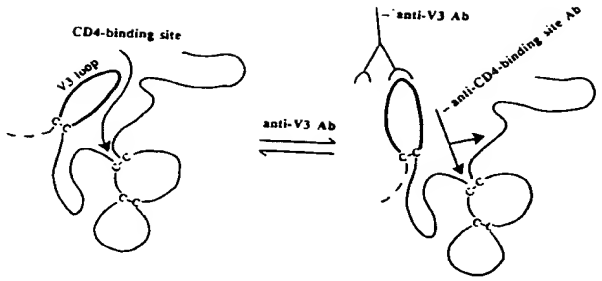

FIG. 3. Hypothetical model explaining enhanced binding to gp120 of an anti-CD4 binding site antibody in the presence of an anti-V3 loop antibody (see Discussion). Additional protein loops of gp120 other than those shown may be involved in CD4 binding.[8]

75%, while increases up to 150% have been seen in other experiments. In contrast, the binding of the biotinylated 4117C to gp160 is not affected by 1125H (Fig. 2).

DISCUSSION

These studies demonstrate that HuMAbs against the two major neutralization epitope clusters of HIV-1 gp120 synergistically neutralize the virus. The mechanism of this synergistic neutralization may be illuminated by binding studies demonstrating enhanced binding of an anti-CD4 binding site HuMAb, 1125H, to recombinant gp160 in the presence of an anti-V3 loop HuMAb, 4117C.

Unidirectional enhancement of binding to viral surface proteins has been observed for pairs of mAbs participating in synergistic neutralization of La Crosse[25] and rubella[26] viruses. The level of binding enhancement which we observe is comparable to that seen by Lussenhop et al.[27] in their analysis of pairs of synergistically neutralizing MAbs against cytomegalovirus. Assuming that the enhanced binding of 1125H induced by 4117C occurs on multiple gp120 molecules on a single virion, it could easily account for the potent synergism observed between 4117C and 1125H in HIV-1 neutralization. This binding enhancement also correlates with the cooperativity in HIV-1 neutralization indicated by the m values obtained from the neutralization curves (Table 1). It should be noted that synergism can be observed without cooperativity,[22] so the fact that we observe cooperativity in this system is likely to be significant in terms of the mechanism of synergy.

One model based on these experiments is that the gp120 molecule assumes at least two different conformations that are in equilibrium with each other. In one conformation (Fig. 3, left), the CD4 binding site is relatively inaccessible, while in the other conformation (Fig. 3, right), it is accessible. The binding of an anti-V3 loop antibody to gp120 favors assumption of the latter conformation, allowing an anti-CD4 binding site antibody greater access to its binding site. A related possibility is that binding of the anti-V3 antibody creates a conformational change in the CD4-binding site such that the affinity of antibodies for this site is increased. The model shown in Figure 3 is attractive because it may explain observations on the human humoral immune response to these neutralizing epitopes. Specifically, it has been observed that individuals infected with HIV-1 produce antibodies against the V3 loop within a few weeks after infection, whereas antibodies against the CD4-binding site typically do not appear for months following infection.[15,28] Our model suggests that the CD4-binding site may become more immunogenic following the production of anti-V3 antibodies in vivo, since the latter's binding to gp120 may increase exposure of epitopes in the CD4-binding site to the immune system.

Interestingly, Sattentau and Moore[29] have observed an enhancement in the binding of anti-V3 loop MAbs to HIV-infected cells at 4°C in the presence of saturating amounts of soluble CD4. This represents a unidirectional binding enhancement in the opposite direction from that which we have observed. The relationship between these observations and ours is unclear, but a number of possible explanations may be given for their differences. Among the latter are that: (1) Sattentau and Moore have shown that gp120 conformation differs at 37 and 4°C;[29] our experiments were done at 37°C while theirs were done at 4°C, (2) Conformational differences may exist between recombinant gp160 and virion- or cell-bound gp120 multimers, and (3) gp120 conformational state equilibria may differ between HIV-1 strains. Further research should clarify these issues.

To our knowledge, the present report details the first observation of synergistic neutralization of HIV by a combination of MAbs and the first observation of synergistic neutralization of any virus by HuMAbs. These results indicate that combinations of HuMAbs against the V3 loop and CD4-binding site epitopes should be much more effective in passive immunotherapy than single HuMAbs. HuMAbs are particularly suited for immunotherapy in humans because of their minimal immunogenicity compared to that of MAbs from a foreign species. In addition, the fact that antibodies against the CD4-binding site and V3 loop may synergistically neutralize HIV-1 in vivo indicates that efforts toward an HIV vaccine should be directed toward including immunogenic epitopes from both of these regions.

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